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Implantation of cocoa butter reduces egg and hatchling size in *Salmo trutta*

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This study demonstrated that, irrespective of hormone type or dose, administering cocoa butter implants during egg development affected the growth of female brown trout *Salmo trutta* and reduced the size of their offspring. Cortisol treatment also increased adult mortality. Caution is urged in the use of implants for studies of maternal hormonal influences on adult fishes and their offspring.

Key words: cortisol; egg development; reproduction; steroid hormone; stream salmonid; testosterone.

INTRODUCTION

A range of different procedures have been used to modify hormone levels of fishes, including incorporation of hormones in the diet, direct injection of hormones dissolved in an oil or saline medium and implantation of osmotic pumps (Gamperl *et al*., 1994). Over the past two decades, cocoa butter implantation has emerged as the preferred technique because it supplies a relatively stable hormone dose over an extended period of time, is comparatively inexpensive, and the treated fishes are thought to be unstressed following recovery from the implantation procedure (Gamperl *et al*., 1994). Correspondingly, injection of chemical-laden butter or oil is now a well-established technique for elevating circulating levels of a range of hormones including cortisol (Eriksen *et al*., 2006), testosterone and 11-ketotestosterone (Pottinger & Pickering, 1985; Sangiao-Alvarellos *et al*., 2006), and compounds that inhibit cortisol synthesis (Doyon *et al*., 2006).

Cocoa butter implants have been most widely used in studies of the effects of elevated cortisol on the biology of fishes. In an early study, Pickering & Duston (1983)

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demonstrated the efficacy of the technique by achieving a three-fold elevation of circulating cortisol levels in 2 year-old brown trout *Salmo trutta* L. 1758 over a 5 week period. Further application of this method has revealed a clear and consistent dose-dependent relationship between cortisol levels and survival, with *S. trutta* exposed to high doses showing greater susceptibility to infection and correspondingly higher mortality rates (Robertson *et al*., 1963; Pickering & Duston, 1983; Carragher *et al*., 1989). Elevated cortisol levels are also associated with poorer body condition and immune function (Pickering *et al*., 1989) and reduced gonad size in both male and female *S. trutta* (Carragher *et al*., 1989). Although these studies have been valuable in identifying the deleterious effects of stress hormones (*i.e.* cortisol) on growth, survival and reproduction in fishes, all have compared the responses of hormone-implanted fishes to those of sham-implanted controls. The aim of this study was to assess whether cocoa butter implants, containing different doses of the steroid hormones testosterone and cortisol, affect the growth and reproduction of female *S. trutta*.

MATERIALS AND METHODS

As part of a broad study on *S. trutta* endocrinology, a cocoa butter pellet was surgically inserted into the intraperitoneal cavity of each of 60 maturing 3 year-old female *S. trutta* obtained from the Howietoun Fishery (University of Stirling, Scotland). Five different implant treatments were prepared ($n = 12$ for each): a sham (containing only cocoa butter), low and high cortisol implants with doses corresponding to 30 and 50 mg kg^{-1} respectively, and low and high testosterone implants with doses corresponding to 2 and 5 mg kg^{-1}. Each of these fish was also tagged with a passive integrated transponder (PIT tag) for individual identification. *Salmo trutta* in an additional control group ($n = 12$) were each implanted with a PIT tag but no cocoa butter.

Hormone dose levels were chosen to elevate circulating hormones within the physiologically relevant range (Pickering *et al*., 1989; Sangiao-Alvarellos *et al*., 2006). Cortisol and testosterone levels for female *S. trutta* both vary in the months prior to spawning, with cortisol reaching peak levels of *c*. 50 ng ml^{-1} at the time of spawning (Pickering & Christie, 1981) and testosterone peaking at 30–50 ng ml^{-1} *c*. 1 month prior to spawning (Pottinger & Pickering, 1987; Norberg *et al*., 1989). Mean ± S.E. ($n = 59$) values from blood samples taken throughout the experimental period were 37 ± 5 ng ml^{-1} for cortisol and 38 ± 3 ng ml^{-1} for testosterone. Implants were prepared following the protocol outlined in Pickering & Duston (1983). Briefly, crystalline cortisol (hydrocortisone 98%, Sigma; www.sigmaaldrich.com) or testosterone (99%, Fluka; www.sigmaaldrich.com) was dissolved in ethanol and mixed with pre-sterilized, molten cocoa butter (melting temperature = 40° C, Mycryo; www.mycryo.com). The ethanol was evaporated by stirring at 75° C and 0·5 ml (*c*. 0·4 g) of the hormone suspension (or cocoa butter only in the case of the sham) was drawn into a 1 ml syringe, allowed to harden and stored at −20° C prior to use.

Fish were implanted by an experienced fish surgeon on 17 September 2009. This date was 2 months prior to the date at which they were predicted to spawn and corresponded with the period of maximum egg yolk deposition (Norberg *et al*., 1989). To administer the implants, fish were anaesthetized using benzocaine (40 mg l^{-1}) and the implant (25 mm long, 4 mm in diameter) was inserted into the intraperitoneal cavity *via* a mid-ventral incision (*c*. 8 mm) posterior to the liver. A PIT tag was also inserted into the cavity. The incision was closed with a single full depth suture (Chromic sterile catgut 3/0 W465, Ethicon; www.ethicon.com) and dusted with an antibiotic wound adhesive (1:1 Cicatrin/Orahesive, Glaxo Smith Kline; www.gsk.com and ConvaTec; www.convatec.co.uk). All fish were given a prophylactic injection of a broad-spectrum antibiotic (Betamox LA, Amoxicillin, at 0·1 ml kg^{-1}, Norbrook Pharmaceuticals; www.norbrook.co.uk) into the dorsal musculature. A similar procedure was

used to implant a PIT tag into each fish in the control group except that the incisions were smaller (*c*. 3 mm) and not sutured.

Fish were measured for fork length (L_F) and body mass (M_T) at the time of implantation, when they ranged in size from 32 to 42 cm and 380 to 860 g. They were maintained at the Almondbank hatchery facility (Perthshire, Scotland) in circular tanks (2 m diameter) and provided with commercial trout pellets *ad libitum*. The fish were housed in groups of 12 individuals mixed equally across treatments, and tanks were supplied with a continuous flow of fresh river water (complete turnover *c*. six times h^{-1}). Weekly mean water temperatures decreased from 9° C at the beginning of the experiment to 2° C when the experiment was terminated in mid-December. Tanks were monitored daily for mortality. No mortality of tagged or implanted fish was observed during, or in the week immediately following, the procedure and samples of fish inspected after 1 week revealed no local inflammatory reaction at the wound sites. Of the samples of fish inspected a month after surgery, all had fully healed wounds.

Following ovulation, which occurred 9–12 weeks post-implantation, females were again weighed and measured and egg samples were obtained by stripping. The total egg mass obtained from each female was recorded (M_E). Growth, in terms of L_F, of individual *S. trutta* was assessed from the change between initial (at time of implantation) and final (at time of spawning) measurements. Absolute growth measurements were converted to growth rates (mm day^{-1}) to account for individual differences in the timing of spawning. Body condition of the *S. trutta* was determined by regressing $\ln(M_T - M_E)$ and $\ln L_F$ at spawning. Relative condition of individual females was assessed from the residuals of this relationship and reflects the mass per length of each female relative to the average observed in the overall sample. After stripping, a sample of *c*. 100 eggs from each female was weighed (accuracy 0·01 g) and preserved in buffered formalin for subsequent determination of mean mass per egg. The remaining eggs were fertilized (one randomly selected male per female) and reared in hatchery trays at ambient temperature (*c*. 0·5 to 2° C overwinter). Hatching occurred between 119 and 133 days post-fertilization and a sample of 10 hatchlings was taken from each batch and preserved in 30% ethanol for subsequent measurement of mass.

ANOVA was used to test the effects of implant presence and hormone type on female growth, body condition and reproductive output, with the last measured as egg and hatchling mass. *Post hoc* tests (Tukey's HSD) and *t*-tests (adjusted for unequal sample size) demonstrated that hormone dose (*i.e*. low compared with high, for either cortisol or testosterone) did not significantly affect the dependent variable in each analysis. Probability values for the test statistics (*P*-values) were greater than 0·5 in all cases and data were pooled across hormone doses in the reported analyses. Correspondingly, the treatment factor in the ANOVA contained four levels: no implant, sham implant, testosterone implant and cortisol implant. Levene's test was used to confirm homogeneity of variance in each analysis ($P > 0{\cdot}2$ for female growth, female condition and egg mass, and $P = 0{\cdot}07$ for hatchling mass). Normal Q-Q plots were also inspected during analysis and confirmed that residuals were normally distributed. All analyses were conducted using the software package R (R Development Core Team, 2008; www.r-project.org). For the analyses of offspring size, female mass was included as a covariate and time to hatching was also included in analyses of hatchling size. Minimal ANOVA models were selected by backwards removal of non-significant terms (α set to 0·05). Tukey's HSD test was used for *post hoc* analyses. A χ^2 goodness-of-fit test was used to assess whether the distribution of observed mortality counts was uniform across treatments.

RESULTS

The administration of implants during the final stages of egg maturation reduced the growth rate (measured as change in L_F) of female *S. trutta* (Table I and Fig. 1). Although observed growth rates were slow, a mean increase in L_F was observed in all treatment groups but was significantly higher in the control compared with implanted females [Fig. 1(a)]. These results indicate that implantation of cocoa butter, irrespective of whether it contained a hormone, reduced growth of maturing female *S. trutta*.

TABLE I. Results of minimal ANOVA models testing the effects of cocoa butter implants containing different steroid hormones on the growth rate (in fork length, L_F, mm day^{-1}) and somatic condition at spawning of female *Salmo trutta*, and on size of eggs and hatchlings obtained from those females. Hatchling age (days to hatching) was not a significant covariate of hatchling size

Effect	d.f.	MS	F	P
Growth rate (L_F) of females				
Treatment	3	0·02	5·1	<0·01
Residual	41	0·06		
Relative condition (residual somatic mass per L_F) of females				
Treatment	3	3289	1·2	0·33
Residual	41	38 396		
Egg mass				
Treatment	3	232	11	<0·001
Female mass	1	322	15	<0·001
Residual	36	770		
Hatchling size				
Treatment	3	195	7·5	<0·001
Female mass	1	137	5·3	<0·05
Residual	28	726		

Conversely, body condition was not significantly different across treatments, although *S. trutta* treated with testosterone and cortisol tended to be in poorer condition at the time of spawning [negative residual somatic condition; Fig. 1(b)]. Additional analyses supported identical outcomes to those described above when using absolute growth (total change in L_F), total change in body mass between implantation and spawning, rate of change of body mass or change in condition factor [Fulton's $K = (M_T - M_E)L_F^{-3}$] instead of growth rate and relative somatic condition.

Congruent with the observed (non-significant) effects on condition, mortality was related to hormone treatment. Of the 19 *S. trutta* that died after implantation, but prior to spawning, 11 were implanted with cortisol and seven were implanted with testosterone; only one fish died in the control group and none in the sham-implanted group. The observed distribution of mortality over the different treatments was significantly less uniform than expected by chance ($\chi^2 = 17$, d.f. $= 3$, $P < 0{\cdot}001$). Standardized residuals (R_S) were calculated for the χ^2 goodness-of-fit test to detect which treatments contributed most strongly to the non-uniform distribution of mortality (Agresti & Finlay, 1986). Mortality was lower than expected in the sham-implanted group ($R_S < -2$) but higher than expected in the cortisol-treated ($R_S > 2$) groups. There was no indication that the dose of the cortisol implants influenced mortality; six of the 11 cortisol-implanted fish that died were from the low dose group. Within the cortisol group, however, mortality was size-dependent with proportionally higher mortality of females that had greater initial mass (Kolmogorov–Smirnov test, $D = 0{\cdot}6$, $P < 0{\cdot}05$). The increase in mortality under cortisol dosing is generally consistent with previous studies (Pickering & Duston, 1983), although previous work has shown dose-dependent effects (Pickering & Pottinger, 1989). Previous studies showing increased mortality associated with cortisol administration have not explored whether such effects are size-dependent and unfortunately the present experiment

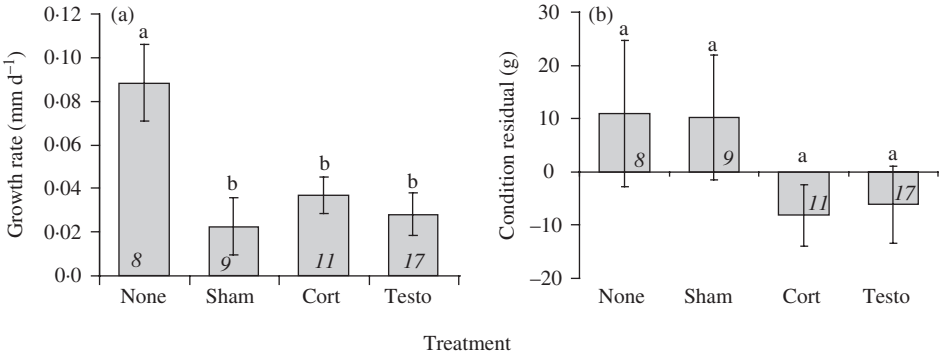

FIG. 1. (a) Growth rate, measured as change in fork length (L_F) per day, and (b) somatic condition of spawning female *Salmo trutta* under four different treatments; controls (none), sham implanted (sham), cortisol implanted (cort) and testosterone implanted (testo). Values in (b) are residuals from a regression of the ln of somatic mass and L_F and represent individual female tissue mass per L_F relative to the mean relationship in the studied population. Values are means ± S.E. Similar lower case letters denote statistically homogeneous sub-sets within growth and condition analyses, and numbers indicate group sample size.

does not illuminate the mechanisms underlying this result. Nevertheless, growth and change in body condition were not associated with initial L_F or M_T (Pearson's correlations, $P > 0{\cdot}2$ in all cases) so size-dependent mortality is unlikely to have influenced the observed treatment effect on *S. trutta* growth (L_F). Finally, the overall distribution of fish mortality demonstrated that mortality was not caused by the surgery procedure: only the cortisol-treated fish showed a level of mortality that was higher than expected.

Eggs from all implanted females, irrespective of hormone type or dose and including the sham group, were significantly smaller than those from control females [Table 1 and Fig. 2(a)]. The minimal model for egg mass included the main effect of treatment and female mass (initial mass at the time of implantation) as a covariate.

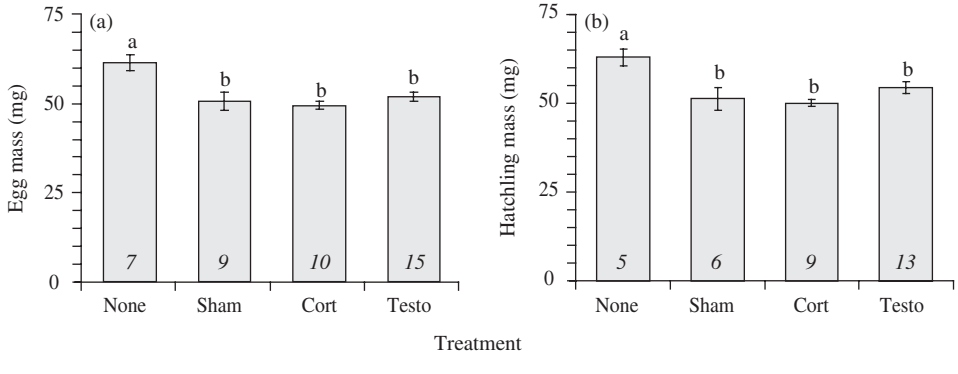

FIG. 2. (a) Mean egg mass and (b) hatchling mass of *Salmo trutta* females under different implant treatments (see Fig. 1 for description of treatments). Lower case letters denote statistically homogeneous subsets; numbers indicate group sample size.

The treatment by female mass interaction was not significant, confirming that the positive correlation between female mass and egg size was consistent across treatments (Table I). The total number of eggs produced by each female did not depend on implant treatment (ANCOVA, $F_{3,34} = 0{\cdot}5$, $P = 0{\cdot}7$), indicating that females did not compensate for reduced egg size by producing more eggs. The observed effect of implantation on egg mass remained consistent throughout embryo development with hatchlings from implanted females also weighing significantly less than controls [Fig. 2(b)]. Female mass at the time of implantation was also a significant predictor of hatchling size (Table I) but hatching time was not retained in the minimal model. Egg mortality over the entire development period was highly variable between groups, ranging from 1 to 57% (median 2%) but was independent of treatment (one-way ANOVA, logit transformed data, $F_{3,33} = 1{\cdot}0$, $P = 0{\cdot}4$).

DISCUSSION

This research indicates that surgical implantation of cocoa butter pellets, regardless of whether the implants contained a hormone, can act as a stressor that suppresses growth of maturing female *S. trutta* and causes a reduction in their offspring size. The available data do not identify the mechanism that underlies these findings. Nevertheless, there are three probable explanations for the results. First, although there was only a small difference in the size of the surgical incision required for implantation of the PIT tag compared to the cocoa butter pellets, there was not an exact control for the surgical technique. The somewhat larger wounds from the implantation procedure (relative to those of the tagged controls) healed quickly but this healing might have required larger energy expenditure than the healing of the smaller wounds of the control fish. Other studies have shown no evidence of effects of surgery itself, or implantation of telemetry tags, on growth rates of fishes even using incisions 1·5 times larger than in this study (Moore *et al*., 1990; Caputo *et al*., 2009). Nevertheless, egg production is energetically expensive (Wootton & Evans, 1976) and the results of this study suggest that reproductive energy allocation may have been constrained by the energetic costs of recovery from surgery. Second, in addition to costs associated with wound healing, an immune response to the presence of foreign organic matter (cocoa butter) may have occurred. There are no data available in the literature to indicate the energetic costs of mounting an immune response in salmonids or fishes in general. Studies on birds and rodents, however, reveal that activation of the immune system can inflate basal metabolic rates by 9–30% (Lochmiller & Deerenberg, 2000; Ots *et al*., 2001; Cutrera *et al*., 2010). Moreover, immune-challenged birds have been shown to lay smaller eggs, indicating that energetic costs of immune responses can affect reproductive output (Cucco *et al*., 2010). Tissue encapsulation of the implants was only observed in a small sub-set of the implanted fish, but such a response may have further restricted energy allocation to tissue growth and egg production and would have manifested over a longer time period than the initial wound healing. Finally, the dissolution of the implants potentially increased circulating levels of oils which may have indirectly reduced egg size by impeding liver function and production of vitellogenin, a protein precursor of egg yolk. For European sea bass *Dicentrarchus labrax* (L. 1758), lipid-enriched diets caused lower vitellogenin levels during the spawning period and a decrease in total reproductive output (Navas *et al*.,

1998). In the present study, the low water temperatures prevented rapid dissolution of the pellets, most of which had lost approximately half of their total mass when recovered at the time of stripping. Nevertheless, it is possible that even a small input of circulating cocoa butter might have suppressed vitellogenin production in the implanted *S. trutta* and caused the observed reduction in egg size.

Mortality of females was not associated with surgery *per se*, being low in both sham implant and control groups and specifically elevated in the cortisol-treated group. Reproducing *S. trutta* are vulnerable because their immune system is suppressed during the spawning phase (Pickering & Christie, 1980). Cortisol implantation has been shown to increase the susceptibility of *S. trutta* to a variety of bacterial and fungal infections (Pickering & Pottinger, 1989). Although the cause of mortality in this experiment could not be determined, many of the female *S. trutta* showed signs of fungal infection during the experimental period. Therefore, the established relationship between cortisol and susceptibility to infection in *S. trutta* (Pickering & Duston, 1983; Pickering & Pottinger, 1989) is the most likely explanation for the heightened mortality of this treatment group. Nevertheless, interpretation of physiological effects of the hormones is confounded by the strong influence of the implant procedure on the eggs, as illustrated by similarities between sham and hormone treatments and differences between sham treatments and controls. Future studies that seek to manipulate egg hormone levels (Eriksen *et al*., 2006) through the use of hormone implants should quantify the effect of the implantation procedure on maternal physiology and on egg size. Interpretation of the results of future studies should also be conducted with cognizance of the potential for the procedure to mask effects of hormone treatment.

Implant procedures are increasingly being used to study the ecology of fishes in the wild (Cooke *et al*., 2003; Gräns *et al*., 2009). Studies validating these methods typically focus on growth rates and survival of the tagged or implanted fishes (Greenstreet & Morgan, 1989; Cooke *et al*., 2003; Jepsen *et al*., 2008). As far as is known, only one study has investigated effects of implants on reproductive physiology, demonstrating that PIT tag presence did not affect gonad development in 0+ year perch *Perca fluviatilis* L. 1758 (Baras *et al*., 2000). In contrast, apparently benign implants, that are very small relative to the size of the fish ($<0{\cdot}2\%$ by mass), significantly affect *S. trutta* reproduction. Therefore, possible effects on reproductive fitness of various tagging and implant procedures commonly used on fishes may require careful consideration in future field and laboratory research.

The role of maternal hormones as mediators of offspring development is of great contemporary interest, particularly because environmental fluctuations experienced during the breeding season induce changes in physiology that are regulated by hormonal mechanisms (Schoech *et al*., 2009), affecting in turn egg composition. The effect of maternal hormones on offspring development is increasingly well understood in avian systems (Groothuis *et al*., 2005). In contrast, knowledge of transgenerational affects of hormones in fish populations remains sparse although recent studies have demonstrated that maternal stress hormones do affect juvenile fish development (McCormick, 1999; Eriksen *et al*., 2006). Hormone implants appear to be a promising technique to manipulate the endocrine system of fishes during reproduction and explore the consequences for offspring development. The strong effects of the surgical and implant procedure, even when performed by a highly experienced surgeon, however, restrict the general applicability of this technique.

In conclusion, these findings suggest that intraperitoneal implantation affects the condition of mature female salmonids and reduces the size of their offspring. Whether this effect is caused by energetic costs of recovering from the implantation procedure, an immune reaction to the presence of foreign matter within the body cavity, or to the chemical properties of cocoa butter itself, the observed changes in egg and hatchling size are likely to have pronounced effects on juvenile performance. Larger eggs produce larger juveniles whose increased competitive ability confers rapid growth and enhanced survival under a range of environmental conditions (Einum & Fleming, 1999). The observed detrimental effects of implants on female *S. trutta* and their offspring mean that potential transgenerational consequences of tagging and implant procedures should be considered in studies of the biology and ecology of fishes.

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